AMENDMENTS TO THE SPECIFICATION:

Please replace the corresponding paragraphs of the specification with the following:

[0002] One of these two regions are known as RD5, as disclosed in Molecular Microbiology (1999), vol. 32, pages 643 to 655 (Gordon S.V. et al.). The other region named RD1-2F9 spans the known region RD1, as disclosed in Molecular Microbiology (1999), vol. 32, pages 643 to 655 (Gordon S.V. et al.). Both of the regions RD1 and RD5, or at least one of them, are absent from the vaccine strains of *M. bovis* BCG and in *M. microti*, strains found involved and used as live vaccines in the 1960's.

[0003] Other applications which are encompassed by the present invention are related to the use of all or part of the said regions to detect virulent strains of ~~*Mycobacteria*~~ *Mycobacteria* and particularly *M. tuberculosis* in humans and animals. The region RD1-2F9 and RD5 are considered as virulence markers under the present invention. The recombinant Mycobacteria and particularly *M. bovis* BCG, after modification of their genome by introduction of all or part of RD1-2F9 region and/or RD5 region in said genome, can be used for the immune system of patients affected with a cancer as for example a bladder cancer.

[0005] The present invention relates to a strain of *M. bovis* BCG or *M. microti*, wherein said strain has integrated all or part of the region RD1-2F9 responsible for enhanced immunogenicity to the tubercle bacilli, especially the genes incoding the ESAT-6 and CFP-10 ~~antigenes~~ antigens. These strains will be referred to as the *M.*

bovis BCG::RD1 or *M. microti*::RD1 strains and are useful as a new improved vaccined for prevention of tuberculosis infections and for treating superficial bladder cancer.

[0021] In a specific aspect, the invention relates to a strain of *M. bovis* BCG or *M. microti* wherein said strain has integrated at least one, two, three or more gene(s) selected from Rv3867 (SEQ ID No 10), Rv3868 (SEQ ID No 11), Rv3869 (SEQ ID No 12), Rv3870 (SEQ ID No 13),Rv3871 (SEQ 1D No 14), Rv3872 (SEQ ID No 15, mycobacterial PE), Rv3873 (SEQ ID No 16, PPE), Rv3874 (SEQ ID No 17, CFP-10), Rv3875 (SEQ ID No 18, ESAT-6), Rv3876 (SEQ ID No 19) and Rv3877 (SEQ ID No 20). In [an] another specific aspect, the invention relates to a strain of *M. bovis* BCG or *M. microti* wherein said strain has integrated at least one, two, three or more gene(s) selected from Rv3871 (SEQ ID No 14), Rv3872 (SEQ ID No 15, mycobacterial PE), Rv3873 (SEQ ID No 16, PPE), Rv3874 (SEQ ID No 17, CFP-10), Rv3875 (SEQ ID No 18, ESAT-6) and Rv3876 (SEQ ID No 19).

[0027] We have shown here that introduction of the RD 1-2F9 region makes the vaccine strains induce a more effective immune response against a challenge with *M. tuberculosis*. However, this first generation of constructs can be followed by other, more fine-tuned generations of constructs, as the complemented BCG::RD1 vaccine strain also showed a more virulent phenotype in severely immuno-compromised (SCID) mice. Therefore, the BCG::RD1 constructs may be modified so as to be applicable as vaccine strains while being safe for immuno-compromised individuals. The term "construct" means an engineered gene unit, usually involving a gene of interest that has been fused to a [promoter] promoter.

[0042] The sequence of the fragment RD1-2F9 (~ 32 kb) covers the region of ~~*the*~~ the *M. tuberculosis* genome AL123456 from ca 4337 kb to ca. 4369 kb, and also contains the sequence described in SEQ ID No 1. Therefore, the invention also embraces *M. bovis* BCG::RD1 strain and *M. microti*::RD1 strain which have integrated the sequence as shown in SEQ ID No 1.

[0076] (A) Map of the *cfp10-esat6* region showing the six possible reading frames and the *M. tuberculosis* H37Rv gene predictions. ~~This map is also available at: (http://genolist.pasteur.fr/TubercuList/)~~.

[0084] (C) Immunoblot analysis of different cell fractions of H37Rv ~~obtained from http://www.cvmbs.colostate.edu/microbiology/tb/ResearchMA.html~~ using either an anti-ESAT-6 antibody or

[0091] **Figure 4:** ~~Immunisation~~ Immunization of mice with BCG::RD1 generates marked ESAT-6 specific T-cell responses and enhanced protection to a challenge with *M. tuberculosis*.

[0092] (A) Proliferative response of splenocytes of C57BL/6 mice ~~immunised~~ immunized subcutaneously (s.c.) with 10^6 CFU of BCG::pYUB412 (open squares) or BCG::RD1-2F9 (solid squares) to *in vitro* stimulation with various concentrations of synthetic peptides from poliovirus type 1 capsid protein VP I, ESAT-6 or Ag85A (K. Huygen, et al., *Infect. Immun.* 62, 363 (1994), L. Brandt, *J. Immunol.* 157, 3527 (1996) and C. Leclerc et al, *J. Virol.* 65, 711 (1991)).

[0094] (C) Concentration of IFN-γ in culture supernatants of splenocytes of C57BL/6 mice stimulated for 72 h with peptides or PPD after s.c. or i.v. ~~immunisation~~ immunization with either BCG::pYUB412 (middle grey and white) or BCG::RDI-2F9 (light grey and black). Mice were inoculated with either 10^6 (white and light grey) or 10^7(middle grey and black) cfu. Levels of IFN-γ were quantified using a sandwich ELISA (detection limit of 500 pg/ml) with the mAbs R4-6A2 and biotin-conjugated XMG1.2. Results are expressed as the mean and standard deviation of duplicate culture wells.

[0095] (D) Bacterial counts in the spleen and lungs of vaccinated and unvaccinated BALB/c mice 2 months after an i.v. challenge with *M. tuberculosis* H37Rv. The mice were challenged 2 months after i.v. inoculation with 10^6 cfu of either BCG::pYUB412 or BCG::RDI-2F9. Organ homogenates for bacterial enumeration were plated on 7H11 medium, with or without hygromycin, to differentiate *M. tuberculosis* from residual BCG colonies. Results are expressed as the mean and standard deviation of 4 to 5 mice and the levels of significance derived using the Wilcoxon ~~rang-sum~~ rank-sum test.

[0101] **Figure 10:** Map of the *M. tuberculosis* H37Rv RD1 genomic region. Map of the fragments used to complement BCG and *M. microti* (black) and the genomic regions deleted from different mycobacterial strains (grey). The middle part shows key genes, putative promoters (P) and transcripts, the various proteins from the RD1 region, their sizes (number of amino acid residues), InterPro domains, and ~~(http://www.ebi.ac.uk/interpro/)~~ membership of *M. tuberculosis* protein families from TubercuList ~~(http://genolist.pasteur.fr/TubercuList/)~~. The dashed lines mark the extent

of the RD1 deletion in BCG, *M. microti* and *M. tuberculosis* clinical isolate MT56 (Brosch, R, *et al.* A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. *Proc Natl Acad Sci U S A* 99, 3684-9. (2002)). *M. bovis* AF2122/97 is shown because it contains a frameshift mutation in Rv3881, a gene flanking the RD1 region of BCG. The fragments are drawn to show their ends in relation to the genetic map, unless they extend beyond the genomic region indicated. pRDI-2F9, pRD1-I106 and pAP35 are based on pYUB412; pAP34 on pKINT; pAP47 and pAP48 on pSM81.

[0102] **Figure 11:** Western blot analysis of various RD1 knock-ins of *M. bovis* BCG and *M. microti.* The left panel shows results of immunodetection of ESAT-6, CFP-10 and PPE68 (Rv3873) in whole cell lysates (WCL) and culture supernatants of BCG; the ~~centre~~ center panel displays the equivalent findings from *M. microti*, and the right panel contains *M. tuberculosis* H37Rv control samples. Samples from mycobacteria transformed with the following plasmids were present in lanes: -, pYUB412 vector control; 1, pAP34; 2, pAP35; 3, RD1-I106; 4, RD1-2F9. The positions of the nearest molecular weight markers are indicated.

[0104] **Figure 13:** Further immunological characterization of responses to BCG::RD1-2F9 **A,** Proliferative response of splenocytes of C57BL/6 mice ~~immunised~~ immunized subcutaneously (s.c.) with 10^6 CFU of BCG::PYUB412 or BCG::RD1-2F9 to *in vitro* stimulation with various concentrations of synthetic peptides from poliovirus type 1 capsid protein VP1 (negative control), ESAT-6 or Ag85A (see Methods for details). **B,** Proliferation of splenocytes from BCG::RD1-2F9-immunised mice in the absence or presence of ESAT-6 1-20 peptide, with or without anti-CD4 or anti-CD8 monoclonal

antibody. Results are expressed as mean and standard deviation of ^{3}H-thymidine incorporation from duplicate wells. ~~e~~ **C**, Concentration of IFN-γ in culture supernatants of splenocytes of C57BL/6 mice stimulated for 72 h with peptides or PPD after s.c. or i.v. ~~immunisation~~ immunization with either BCG::pYUB412 or BCG::RD1-2F9. Mice were inoculated with either 10^6 or 10^7 CFU. Results are expressed as the mean and standard deviation of duplicate culture wells.

[0105] **Figure 14:** Mouse protection studies. **A,** Bacterial counts in the spleen and lungs of vaccinated and unvaccinated C57BL/6 mice 2 months after an i.v. challenge with *M. tuberculosis* H37Rv. The mice were challenged 2 months after i.v. inoculation with 10^6 cfu of either BCG::pYUB412 or BCG::RD1-2F9. Organ homogenates for bacterial enumeration were plated on 7H11 medium, with or without hygromycin, to differentiate *M. tuberculosis* from residual BCG colonies. Results are expressed as the mean and standard deviation of 4 mice. Hatched columns correspond to the cohort of unvaccinated mice, while white and black columns correspond to mice vaccinated with BCG::pYUB412 and BCG::RD1-2F9, respectively. **B,** Bacterial counts in the spleen and lungs of vaccinated and unvaccinated C57BL6 mice after an aerosol challenge with 1000 CFUs of *M. tuberculosis*. All mice were treated with antibiotics for three weeks prior to infection with *M. tuberculosis*. Data are the mean and SE measured on groups of three animals, and differences between groups were ~~analysed~~ analyzed using ANOVA (*$p<0.05$, **$p<0.01$).

[0106] **Figure 15:** Guinea pig protection studies. **A,** Mean weight gain of vaccinated and unvaccinated guinea pigs following aerosol infection with *M.*

tuberculosis H37Rv. Guinea pigs were vaccinated with either saline (triangles), BCG (squares) or BCG::RD1-2F9 (filled circles). The error bars are the standard error of the mean. Each time point represents the mean weight of six guinea pigs. For the saline vaccinated group the last live weight was used for calculating the means as the animals were killed on signs of severe tuberculosis which occurred after 50, 59, 71, 72, 93 and 93 days. **B,** Mean bacterial counts in the spleen and lungs of vaccinated and unvaccinated guinea pigs after an aerosol challenge with *M. tuberculosis* H37Rv. Groups of 6 guinea pigs were vaccinated subcutaneously with either saline, BCG or BCG::RD1-2F9 and infected 56 days later. Vaccinated animals were killed 120 days following infection and unvaccinated ones on signs of suffering or significant weight loss. The error bars represent the standard error of the mean of six observations. **C,** Spleens of vaccinated guinea pigs 120 days after infection with *M. tuberculosis* H37Rv; left, animal ~~immunised~~ immunized with BCG; right, animal ~~immunised~~ immunized with BCG::RD1-2F9.

[0107] **Figure 16:** Diagram of the *M. tuberculosis* H37Rv genomic region showing a working model for biogenesis and export of ESAT-6 proteins. It presents a possible functional model indicating predicted subcellular localization and potential interactions within the mycobacterial cell envelope. Rosetta stone analysis indicates direct interaction between proteins Rv3870 and Rv3871, and the sequence similarity between the N-terminal domains of Rv3868 and Rv3876 suggests that these putative chaperones might also interact. Rv3868 is a member of the AAA-family of ATPases that perform chaperone-like functions by assisting in the assembly, and disassembly of

protein complexes (Neuwald, A.F., Aravind, L., Spouge, J.L. & Koonin, E.V. AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Res* **9**, 27-43. (1999).). It is striking that many type III secretion systems require chaperones for ~~stabilisation~~ stabilization of the effector proteins that they secrete and for prevention of premature protein-protein interactions (Page, A.L. & Parsot, C. Chaperones of the type III secretion pathway: jacks of all trades. *Mol Mcrobiol* **46**, 1-11. (2002).). Thus, Rv3868, and possibly Rv3876, may be required for the folding and/or ~~dimerisation~~ dimerization of ESAT-6/CFP-10 proteins (Renshaw, P.S., *et al.* Conclusive evidence that the major T-cell antigens of the M. tuberculosis complex ESAT-6 and CFP-10 form a tight, 1:1 complex and characterisation of the structural properties of ESAT-6, CFP-10 and the ESAT-6-CFP-10 complex: implications for pathogenesis and virulence. *J Biol Chem* **8**, 8 (2002).), or even to prevent premature ~~dimerisation~~ dimerization. ESAT-6/CFP-10 are predicted to be exported through a transmembrane channel, consisting of at least Rv3870, Rv3871, and Rv3877, and possibly Rv3869, in a process catalysed by ATP-hydrolysis. Rv3873 (PPE 68) is known to occur in the cell envelope and may also be involved as shown herein.

[0113] When used as a subunit vaccine, ESAT-6 elicits T-cell responses and induces levels of protection weaker than but akin to those of BCG (L. Brandt et al, *Infect. Immun.* **68**, 791 (2000)). Challenge experiments were conducted to determine if induction of immune responses to BCG::RD1-encoded antigens, such as ESAT-6, could improve protection against infection with *M. tuberculosis*. Groups of mice inoculated

with either BCG::pYUB412 or BCG::RD1 were subsequently infected intravenously with *M. tuberculosis* H37Rv. These experiments showed that ~~immunisation~~ immunization with the BCG::RD1 "knock-in" inhibited the growth of *M. tuberculosis* within both BALB/c (Fig. 4D) and C57BL/6 mice when compared to inoculation with BCG alone.

[0120] End-sequencing reactions were performed with a *Taq* DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems) using a mixture of 13 :1 of DNA solution, 2 :1 of Primer (2 M) (SP6-BAC1, AGTTAGCTCACTCATTAGGCA (SEQ ID No 15), or T7-BAC1, GGATGTGCTGCAAGGCGATTA (SEQ ID No 16)), 2.5 :1 of Big Dye and 2.5 :1 of a 5X buffer (50 mM $MgCl_2$, 50 mM Tris). Thermal cycling was performed on a PTC-100 amplifier (MJ Inc.) with an initial denaturation step of 60 s at 95°C, followed by 90 cycles of 15 s at 95°C, 15 s at 56°c, 4 min at 60°C. DNA was then precipitated with 80 :1 of 76% ethanol and centrifuged at 3000 rpm for 30 min. After discarding the supernatant, DNA was finally rinsed with 80 µl of 70% ethanol and resuspended in appropriate buffers depending on the type of automated sequencer used (ABI 377 or ABI 3700). Sequence data were transferred to Digital workstations and edited using the TED software from the Staden package (37). Edited sequences were compared against the *M. tuberculosis* H37Rv database ~~(http://genolist.pasteur.fr/TubercuList/)~~, the *M. bovis* BLAST server ~~(http://www-sanger.ac.uk/Projects/M_bovis/blast_server.shtml)~~, and in-house databases to determine the relative positions of the M. naicroti OV254 BAC end-sequences.

[0130] Antigens ESAT-6 and CFP-10 are absent from *M. microti.* One of the most interesting findings of the BAC to BAC comparison was a novel deletion in a

genomic region close to the origin of replication (~~figure~~ Figure 5). Detailed PCR and sequence analysis of this region in *M. microti* OV254 showed a segment of 14 kb to be missing (equivalent to *M. tuberculosis* H37Rv from 4340,4 to 4354,5 kb) that partly overlapped $RD1^{bcg}$ absent from *M. bovis* BCG. More precisely, ORFs Rv3864 and Rv3876 are truncated in *M. microti* OV254 and ORFs Rv3865 to Rv3875 are absent (~~figure~~ Figure 6). This observation is particularly interesting as previous comparative genomic analysis identified $RD1^{bcg}$ as the only RD region that is specifically absent from all BCG sub-strains but present in all other members of the *M. tuberculosis* complex (1, 4, 13, 29, 35). As shown in Figure 6, in *M. microti* OV254 the $RD1^{mic}$ deletion is responsible for the loss of a large portion of the conserved ESAT-6 family core region (40) including the genes coding for the major T-cell antigens ESAT-6 and CFP-10 (2, 15). The fact that previous deletion screening protocols employed primer sequences that were designed for the right hand portion of the $RD1^{bcg}$ region (i.e. gene Rv3878) (6, 39) explains why the $RD1^{mic}$ deletion was not detected earlier by these investigations. Figure 6 shows that $RD1^{mic}$ does not affect genes Rv3877, Rv3878 and Rv3879 which are part of the $RD1^{bcg}$ deletion.

[0134] Lack of MiD1 provides genomic clue for *M. microti* OV254 characteristic spoligotype. MiD1 encompasses the three ORFs Rv2816, Rv2817 and Rv2818 that encode putative proteins whose functions are yet unknown, and has occurred in the direct repeat region (DR), a polymorphic locus in the genomes of the tubercle bacilli that contains a cluster of direct repeats of 36 bp, separated by unique spacer sequences of 36 to 41 by (17), (~~figure~~ Figure 7). The presence or absence of 43 unique spacer

sequences that intercalate the DR sequences is the basis of spacer-oligo typing, a powerful typing method for strains from the *M. tuberculosis* complex (23). *M. microti* isolates exhibit a characteristic spoligotype with an unusually small DR cluster, due to the presence of only spacers 37 and 38 (43). In *M. microti* OV254, the absence of spacers 1 to 36, which are present in many other *M. tuberculosis* complex strains, appears to result from an IS*6110* mediated deletion of 636 by of the DR region. Amplification and *Pvu*II restriction analysis of a 2.8 kb fragment obtained with primers located in the genes that flank the DR region (Rv2813c and Rv2819) showed that there is only one copy of IS*6110* remaining in this region (~~figure~~ Figure 7). This IS*6110* element is inserted into ORF Rv2819 at position 3,119,932 relative to the *M. tuberculosis* H37Rv genome. As for other IS*6110* elements that result from homologous recombination between two copies (7), no 3 base-pair direct repeat was found for this copy of IS*6110* in the DR region. Concerning the absence of spacers 39-43 (~~figure7~~ Figure 7), it was found that *M. microti* showed a slightly different organization of this locus than *M. bovis* strains, which also characteristically lack spacers 39-43. In *M. microti* OV254 an extra spacer of 36 bp was found that was not present in *M. bovis* no in *M. tuberculosis* H37Rv. The sequence of this specific spacer was identical to that of spacer 58 reported by van Embden and colleagues (42). In their study of the DR region, in many strains from the *M. tuberculosis* complex, this spacer was only found in *M. microti* strain NLA000016240 (AF189828) and in some ancestral *M. tuberculosis* strains (3, 42). Like MiD1, MiD2 most probably results from an IS*6110*-

mediated deletion of two genes (Rv3188, Rv3189) that encode putative proteins whose function is unknown (Table 3 above and Table 4 below).

[0144] We have searched for major genomic variations, due to insertion-deletion events, between the vole pathogen, *M. microti*, and the human pathogen, *M. tuberculosis*. BAC based comparative genomics led to the identification of 10 regions absent from the genome of the vole bacillus *M. microti* OV254 and several insertions due to IS*6110*. Seven of these deletion regions were also absent from eight other *M. microti* strains, isolated from voles or humans, and they account for more than 60 kb of genomic DNA. Of these regions, $RD1^{mic}$ is of particular interest, because absence of part of this region has been found to be restricted to the BCG vaccine strains to date. As *M. microti* was originally described as non pathogenic for humans, it is proposed here that RD1 genes ~~is~~ are involved in the pathogenicity for humans. This is reinforced by the fact that $RD1^{bcg}$ (29) has lost putative ORFs belonging to the *esat*-6 gene cluster including the genes encoding ESAT-6 and CFP-10 (Fig. 6) (40). Both polypeptides have been shown to act as potent stimulators of the immune system and are antigens recognized during the early stages of infection (8, 12, 20, 34). Moreover, the biological importance of this RD1 region for mycobacteria is underlined by the fact that it is also conserved in *M. leprae*, where genes ML0047-ML0056 show high similarities in their sequence and operon organization to the genes in the *esat-6* core region of the tubercle bacilli (11). In spite of the radical gene decay observed in *M. leprae*, the *esat-6* operon apparently has kept its functionality in this organism.

[0156] To assess the effect of the RD1^{mic} deletion of *M. microti* on the export of ESAT-6 and CFP-10 and subsequent antigen handling, the experiments were replicated in this genomic background. As with BCG, ESAT-6 and CFP-10 were only exported into the supernatant fraction in significant amounts if expressed in conjunction with the entire *esx* cluster (Fig. 11). The combined findings demonstrate that complementation with *esxA* or *esxB* alone is insufficient to produce a recombinant vaccine that secretes these two antigens. Rather, secretion requires expression of genes located both upstream and downstream of the antigenic core region confirming our hypothesis[20] that the conserved *esx* gene cluster does indeed encode functions essential for the export of ESAT-6 and CFP-10.

[0158] Since the classical observation that inoculation with live, but not dead BCG, confers protection against tuberculosis in animal models it has been considered that secretion of antigens is critical for maximizing protective T-cell immunity. Using our panel of recombinant vaccines we were able to test if antigen secretion was indeed essential for eliciting ESAT-6 specific T-cell responses. Groups of C57/BL6 mice were inoculated subcutaneously with one of six recombinant vaccines (BCG-pAP47, BCG-pAP48, BCG::RD1-pAP34, BCG::RD1-pAP35, BCG::RD1-I106, BCG::RD1-2F9) or with BCG transformed with the empty vector pYUB412. Three weeks following vaccination, T-cell immune responses to the seven vaccines were assessed by comparing antigen-specific splenocyte proliferation and gamma interferon (IFN-γ) production (Fig. 12A). As anticipated, all of the vaccines generated splenocyte proliferation and IFN-γ production in response to PPD (partially purified protein derivative) but not against an unrelated

MalE control peptide, indicating successful vaccination in each case. However, only splenocytes from the mice inoculated with BCG::RD1-2F9 proliferated markedly in response to the inununodominant ESAT-6 peptide (Fig. 12A). Furthermore, ~~IFN—~~ IFN-γ was only detected in culture supernatants of splenocytes from mice immunized with BCG::RD1-2F9 following incubation with the ESAT-6 peptide (Fig. 12B) or recombinant CFP-10 protein (data not shown). These data demonstrate that export of the antigens is essential for stimulating specific Th1-oriented T-cells.

[0159] Further characterization of the immune responses was carrid out. Splenocytes from mice immunized with BCG::RD1-2F9 or control BCG both proliferated in response to the immunodominant antigen 85A peptide (Fig. 13A). The strong splenocyte proliferation in the presence of ESAT-6 was abolished by an anti-CD4 monoclonal antibody but not by anti-CD8 indicating that the $CD4^+$ T-cell subset was involved (Fig. 13B). Interestingly, as judged by *in vitro* IFN-γ response to PPD and the ESAT peptied, subcutaneous immunization generated much stronger T-cell responses (Fig. 13C) compared to intravenous injection. After subcutaneous ~~immunisation~~ immunization with BCG::RD1-2F9, strong ESAT-6 specific resposnes were also detected in inguinal lymph nodes (data not shown). These experiments demonstrated that the ESAT-6 T-cell immune responses to vaccination with BCG::RD1-2F9 were potent, reproducible and robust, making this recombinant an excellent candidate for protection studies.

[0164] **4.2 Results.** Although experiments in mice convincingly demonstrated a superior protective efficacy of BCG::RD1 over BCG it was important to establish a

similar effect in the guinea pig model of tuberculosis. Guinea pigs are exquisitely sensitive to tuberculosis, succumbing rapidly to low dose infection with *M. tuberculosis*, and develop a necrotic granulomatous pathology closer to that of human tuberculosis. Immunization of guinea pigs with BCG::RD1-2F9 was therefore compared to conventional BCG vaccination. Groups of six guinea pigs were inoculated subcutaneously with saline, BCG or BCG::RD1-2F9. Eight weeks following inoculation the three guinea pig cohorts were challenged with *M. tuberculosis* H37Rv via the aerosol route. Individual animals were weighed weekly and were killed 17 weeks after challenge or earlier if they developed signs of severe tuberculosis. Whereas all unvaccinated guinea pigs failed to thrive and were euthanised before the last time-point because of overwhelming disease, both the BCG- and recombinant BCG::RD1-2F9-vaccinated animals progressively gained weight and were clinically well when killed on termination of the experiment (Fig. 15A). This indicated that although the BCG::RD1-2F9 recombinant is more virulent in severely immunodeficient mice (Pym, A.S., Brodin, P., Brosch, R, Huerre, M. & Cole, S.T. Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines *Mycobacterium bovis* BCG and *Mycobacterium micron. Mol. Microbiol.* 46, 709-717 (2002)), there is no increased pathogenesis in the highly susceptible guinea pig model of tuberculosis. Moreover, when the bacterial loads in the spleens of the vaccinated animals were compared there was a greater than ten-fold reduction in the number of CFU recovered from the animals immunised with BCG::RD1-2F9 when compared to BCG (Fig. 15B). Interestingly, there was no significant difference between the number of CFU obtained from the lungs of the two vaccinated

groups indicating that the organ-specific enhanced protection observed in mice vaccinated with BCG::RD1-2F9 was also seen with guinea pigs. This marked reduction of bacterial loads in the spleens of BCG::RD1-2F9 ~~immunised~~ immunized animals was also reflected in the gross pathology. Visual examination of the spleens showed that tubercules were much larger and more numerous on the surface of the BCG-vaccinated guinea pigs (Fig. 15C). These results demonstrate that the recombinant vaccine BCG::RD1-2F9 conveys enhanced protection to an aerosol challenge with *M. tuberculosis* in two distinct animal models.

[0167] Tuberculosis is still one of the leading infectious causes of death in the world despite a decade of improving delivery of treatment and control strategies (Dye, C., Scheele, S., Dolin, P., Pathania, V. & Raviglione, M.C. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. *Jama* **282**, 677-86. (1999)). Reasons for the recalcitrance of this pandemic are multi-factorial but include the modest efficacy of the widely used vaccine, BCG. Two broad approaches can be distinguished for the development of improved tuberculosis vaccines (Baldwin,. S.L., *et al.* Evaluation of new vaccines in the mouse and guinea pig model of tuberculosis. *Infection & Immunity* 66, 2951-9 (1998), Kaufmann, S.H. How can immunology contribute to the control of ~~tuberculosis *Nature*~~ tuberculosis *Nature Rev Inmunol* **1**, 20-30.(2001), and Young, D.B. & Fruth, U. in *New Generation Vaccines* (eds. Levine, M., Woodrow, G., Kaper, J. & Cobon GS) 631-645 (Marcel Dekker, 1997)). These are the development of subunit vaccines based on purified protein antigens or new live vaccines that stimulate

a broader range of immune responses. Although a growing list of individual or combination subunit vaccines, and hybrid proteins, have been tested, none has yet proved superior to BCG in animal models (Baldwin, S.L., *et al.*, 1998). Similarly, new attenuated vaccines derived from virulent *M. tuberculosis* have yet to out-perform BCG (Jackson, M., *et al.* Persistence and protective efficacy of a *Mycobacterium tuberculosis* auxotroph vaccine. *Infect Immun* **67**, 2867-73. (1999) and Hondalus, M.K, *et al.* Attenuation of and protection induced by a leucine auxotroph of *Mycobacterium tuberculosis. Infect Immun* **68**, 2888-98. (2000)). Interestingly, the only vaccine that appears to surpass BCG is a BCG recombinant over expressing antigen 85A (Horwitz, M.A., Harth, G., Dillon, B.J. & Maslesa-Galic, S. Recombinant bacillus calmette-guerin (BCG) vaccines expressing the Mycobacterium tuberculosis 30-kDa major secretory protein induce greater protective immunity against tuberculosis than conventional BCG vaccines in a highly susceptible animal model. *Proc Natl Acad Sci USA* **97**, 13853-8. (2000)). The basis for this vaccine was the notion that over-expression of an immunodominant T-cell antigen could quantitatively enhance the BCG-elicited immune response.

[0170] The antigen genes, *esxBA*, lie at the ~~centre~~ center of the conserved gene cluster. Bioinformatics and comparative genomics predicted that both the conserved upstream genes Rv3868-Rv3871, as well as the downstream genes Rv3876-Rv3877, would be required for secretion (Fig. 1) and strong experimental support for this prediction is provided here. Our experiments show that only when BCG or *M. microti* are complemented with the entire cluster is maximal export of ESAT-6 and CFP-10

obtained. This suggests that at least Rv3871 and either Rv3876 or Rv3877 are indeed essential for the normal secretion of ESAT-6 as these are the only conserved genes absent or disrupted in BCG which are not complemented by RD1-I106 or RD1-pAP35. These genes encode a large transmembrane protein with ATPase activity, an ATP-dependent chaperone and an integral membrane protein, functional predictions compatible with them being part of a multi- protein complex involved in the translocation of polypeptides. Amongst the proteins encoded by the *esx* cluster, Rv3871 and Rv3877 are highly conserved, as orthologues have been identified in the more streamlined clusters found in other actinomycetes, further supporting their direct role in secretion (Gey Van Pittius, N.C., *et al.* The ESAT-6 gene cluster of *Mycobacterium tuberculosis* and other high G+C Gram-positive bacteria. *Genome Biol* **2**, 44.1-44.18 (2001)). It has been shown recently that ESAT-6 and CFP-10 form a heterodimer *in vitro* (Renshaw, P.S.; *et al.* Conclusive evidence that the major T-cell antigens of the *M. tuberculosis* complex ESAT-6 and CFP-10 form a tight, 1:1 complex and characterisation of the structural properties of ESAT-6, CFP-10 and the ESAT-6-CFP-10 complex: implications for pathogenesis and virulence. *J Biol Chem* 8, 8 (2002)) but it is not known whether ~~dimerisation~~ dimerization precedes translocation across the cell membrane or occurs at a later stage *in vivo*. In either case, chaperone or protein clamp activity is likely to be required to assist dimer formation or to prevent premature complexes arising as is well documented for type III secretion systems (Page, A.L. & Parsot, C. Chaperones of the type III secretion pathway: jacks of all trades. *Mol Microbiol* **46**, 1-11. (2002)). These, and other questions concerning the precise roles of the individual components of the

ESAT-6 secretory apparatus, can now be addressed experimentally using the tools developed here.

[0173] Recombinant BCG vaccines have definite advantages over other vaccination strategies in that they are inexpensive, ~~easey~~ easy to produce and convenient to store. However, despite an unrivalled and enviable safety record, concerns remain, and BCG is currently not administered to individuals with HIV infection. As shown above, the recombinant BCG::RD1-2F9 grows more rapidly in Severe Combined Immunodeficiency (SCID) mice, an exreme model of immunodeficiency, than its parental BCG strain. However, in both immunocompetent mice and guinea pigs we have not observed any increased pathology, only a slight increase in persistence which may be beneficial, since the declining efficacy of BCG with serial passage has been attributed to an indavertent increase in its attenuation (Behr, M.A. & Small, P.M. Has BCG attenuated to ~~impotence *Nature*~~ impotence? *Nature* 389, 133-4. (1977)).

At [0046], please change the formatting to remove the indentation of the entire paragraph.

Replacement drawings are attached hereto and incorporated herein by reference.